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Expression of granzyme A and B proteins by cytotoxic lymphocytes involved in acute renal allograft rejection

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Expression of granzyme A and B proteins by cytotoxic lymphocytes involved in acute renal allograft rejection. Granzymes A and B are serine-proteinases stored in the granules of activated cytotoxic T-lymphocytes and natural killer (NK) cells. Expression of granzymes in tissues can be used as an activation marker for cytotoxic cells. Using mAbs specific for human granzyme A or B in immunohistochemical staining techniques we investigated expression of granzyme A and B by lymphocytes infiltrating acutely rejected renal allografts. Twelve core needle biopsies were taken from ten different patients during an episode of acute rejection. Eleven biopsies contained high numbers of granzyme A and B positive lymphocytes infiltrating tubular epithelium, and vascular and glomerular structures. In one patient infiltrating lymphocytes did not express granzyme A and only low amounts of granzyme B. No correlation was found between the number of granzyme positive cells and the severity of the rejection as classified by conventional histological criteria. In one tissue specimen from a patient with a renal allograft without signs of rejection, the number of granzyme positive cells was much lower compared to that of the transplant group. In spite of the presence of a marked inflammatory infiltrate, no granzyme positive cells were detected in renal biopsies from patients with various inflammatory, not transplant-related, renal diseases. Phenotypic analysis showed that granzymes A and B were expressed by $CD56^+$ NK cells and $CD3^+$ cells, representing cytotoxic T-lymphocytes. Thus, this study demonstrates that granzyme A and B protein-expressing lymphocytes infiltrate the kidney allografts during an acute cellular rejection but not in several other inflammatory renal diseases. The appearance of these granzymes could therefore provide a means to predict a rejection episode or to monitor the efficacy of immunosuppressive therapy.

Despite consideral improvement of immunosuppressive medication, in particular the introduction of cyclosporin A, renal transplantation is still confronted with several complications which may affect graft functioning. Apart from surgical and urological complications, early allograft dysfunction may result from several processes, such as cyclosporin A toxicity, acute tubular necrosis (ATN) or immunologic rejection of the allograft. A main reason for allograft rejection is the presence of incompatible major histocompatibility (MHC) class I and II antigens on the grafted tissue, which are the major targets for an immunological attack. These so-called allo-MHC antigens are presented to $CD4^+$ T cells, which subsequently become activated and induce clonal expansion of antigen activated $CD4^+$ and $CD8^+$ cells [reviewed in 1].

Damage of the allograft tissue is frequently mediated by activated cytotoxic cells. Several cell populations may mediate cellular cytotoxicity, including allospecific $CD3^+$ $CD8^+$ cytotoxic T lymphocytes (CTL), $CD3^+CD4^+$ T helper cells [1] and natural killer (NK) cells which are involved in non-MHC restricted cytotoxicity [2, 3]. To assess the contribution of these cell types in renal allograft rejection, many studies have analyzed the phenotype of the different lymphocyte subsets present in the allograft [4, 5]. Detection of lymphocyte infiltration in this way, however, may not always provide information about the activation state of these cells, although the expression of activation markers, such as IL-2 receptor-β (CD25) and MHC class II [6, 7] or proliferation characteristics of the lymphocytes have been used in this regard [8].

Cytoplasmic granules of activated CTL and NK cells contain a set of specific molecules which play a role in the destruction of target cells. Upon recognition of the target cell, reorientation and subsequent release of these granule contents towards the target cell occurs [9, 10]. These contents consist of a pore forming protein "perforin" [11, 12] and a group of highly homologous serine proteinases, termed granzymes. In humans, two granzymes, A and B, have been characterized at the cDNA as well as at the protein level [9, 13, 14]. The presence of both perforin and granzyme A correlates with activation and subsequent cytolytic potential of these cells *in vitro* [15–17]. Recently, studies using *in situ* hybridization have shown *in vivo* expression of granzyme and perforin mRNA by activated cytotoxic cells in murine [18–20] and human [21–23] tissues. In biopsies from patients who had undergone cardiac transplantation, granzyme A and perforin mRNA positive lymphocytes were specifically associated with a rejection episode [21]. Moreover, the granzyme B gene appeared to be expressed in human renal allografts biopsies from patients with an acute cellular rejection, as was assessed by a semi-quantitative polymerase chain reaction (PCR) analysis [24].

Recently, we have described the production of two sets of monoclonal antibodies (mAb) against human granzyme A and B, respectively [25]. In this study we have used these mAb to detect expression of granzyme proteins by activated cytotoxic cells in renal allograft biopsies showing an acute cellular rejection. A

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Table 1. Clinical characteristics of the patients studied

Patient	Age/Sex	Tx/Bx interval	Diagnosis
1	52 M	1 month	ACR grade III
2	34 M	2 months	ACR grade II
3	58 F	3 weeks	ACR grade II
4	25 M	10 months	ACR grade II; also chronic damage
5	46 M	2 weeks	ACR grade I–II
6c	40 M	3 months	ACR grade I–II
6b	40 M	6 weeks	ACR grade II
6a	40 M	3 weeks	ACR grade III
7	41 F	1 month	ACR grade II; also chronic damage
8	60 F	3 weeks	ACR grade III
9	43 M	6 months	ACR grade II–III
10	44 M	4 days	ACR grade II–III
Control s			
11	69 F	—	Chronic pyelonephritis
12	53 M	—	Adenocarcinoma renis
13	36 M	4 months	Focal glomerulosclerosis in renal Tx
14	56 M	—	Arteriosclerosis in DM I
15	64 M	—	Vasculitis
16	20 M	—	Granulomatous inflammation
17	55 M	—	Interstitial nephritis

Note that patient 6 was studied during 3 different rejection episodes. For diagnosis, as determined by routine histological examination, see **Methods.** Tx/Bx interval is the interval between time of transplantation and time of biopsy.

double immunohistochemical staining was used to identify the phenotype of these cytotoxic cells.

Methods

Patients

All renal allograft patients received basic immunosuppressive drug therapy consisting of prednisolone (10 mg/day) and cyclosporin A (yielding whole blood trough levels of 150 to 200 μg/liter) orally. Twelve core needle biopsies were taken from 10 different patients during an episode of acute rejection. Rejection was clinically defined as an insufficient improvement of renal function, or a rise of an initially decreasing or stable serum creatinine, with or without a decrease in urinary output and subsequently recovery after therapy. As controls, biopsies and nephrectomy specimens from seven patients with different renal diseases were included (Table 1).

A portion of each tissue was fixed for three hours in a solution containing 50 mM sodium phosphate buffer, 40 mM acetic acid, 60% (vol/vol) ethanol, 3% (vol/vol) formaldehyde, pH 3.8 [according to Tellyesniczky (TEL)], and embedded in paraffin for routine examination and immunohistochemical studies of expression of granzymes A and B. An additional part of the specimen from six biopsies was snap frozen in liquid nitrogen for double immunohistochemical staining. Cryostat sections (5 μm) were placed on poly-L-lysine coated glass slides, air dried, fixed in aceton for 10 minutes at room temperature (RT) and stored at −70°C until immunohistochemical analysis.

Classification

Histological diagnosis of acute cellular rejection was based on the following parameters according to the Banff classification [26]: "mononuclear infiltration of the interstitium; signs of tubulitis; or vascular endothelitis." The biopsies were classified into three categories according to the severity of the rejection: grade I, II and III. In some cases (patients 4 and 9), these biopsies contained elements of chronic damage, as manifested by the presence of ischemic leasions, interstitial fibrosis or tubular atrophy. There were no signs of vascular rejection or active cytomegalovirus infection at the time of biopsy.

Antibodies

Monoclonal antibodies specific for human granzymes A and B, respectively, were elicited against recombinant human granzyme A and granzyme B proteins as described elsewhere [25]. We have previously shown that on immunoblot the mAbs GrA-6 and GrA-8 (both IgG1) react specifically with granzyme A, and mAb GrB-4 (IgG1) with granzyme B from IL-2-stimulated peripheral blood mononuclear cells. In addition, in immunofluorescense and immunohistochemical studies, both mAbs specifically detected human granzymes A and B in cytotoxic granules from NK cells [25], CTL clones and activated peripheral blood lymphocytes (manuscript in preparation). The mAbs were purified by protein G affinity-chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden). Biotinylation of antibodies was carried out using long chain biotinyl-N-hydroxysuccinimide ester sulfonacid (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's instructions. Fluorescein isothyocyanate (FITC)-labeling of GrB-4 was performed essentially as described previously [27].

The following FITC-labeled mAbs were used to determine the phenotype of the T lymphocytes: anti-Leu-4, anti-Leu-3a+b, obtained from Becton Dickinson (San Jose, CA, USA), directed against CD3 and CD4, respectively, and Dako-T8, obtained from Dakopatts (Copenhagen, Denmark), directed against CD8. NK cells were detected using mAb 733.24 against CD56 (NKH1 marker), kindly provided by Dr. E. Roosnek (Department of Transplantation, Hospital Cantonal Genève, Switzerland). Rabbit polyclonal antibodies against human CD3 (Dakopatts) was used to detect T cells in paraffin embedded tissue. Biotinylated rabbit anti-mouse Fab_2 immunoglobulins, biotinylated swine anti-rabbit Fab_2 immunoglobulins, rabbit anti-FITC immunoglobulins, alkaline phosphatase (AP)-conjugated swine anti-rabbit immunoglobulins and horseradish (HRP)-labeled streptavidin-ABC complex were all purchased from Dakopatts.

Immunohistochemical techniques

Monostaining. Paraffin embedded tissue sections (4 μm) were used to detect granzyme A, B and CD3 expressing cells infiltrating the renal tissue. All incubations were performed at RT, after each incubation step the slides were washed three times for five minutes with phosphate buffered saline (PBS).

Paraffin sections were deparaffinized and incubated with methanol containing 0.3% (vol/vol) H_2O_2 (Sigma, St. Louis, MO, USA) for 30 minutes to inactivate endogenous peroxidase. Then, slides were washed and treated with 10 mM sodium citrate, pH 6.0, for 10 minutes at 100°C, to enhance the immunoreactivity of the anti-granzyme mAbs, or incubated with 0.25% (wt/vol) pepsine in 10 mM HCl for 15 minutes at 37°C for the detection of CD3. Slides were rinsed and pre-incubated with normal goat serum (1:10 diluted in PBS-1% (wt/vol) bovine serum albumin, PBS-B) for 10 minutes and subsequently incubated for one hour with the GrA-6, GrB-4 mAb (both 10 μg/ml in PBS-B) or polyclonal antibodies against CD3. Sections were washed and then incubated for 30 minutes with biotinylated rabbit anti-mouse Fab_2 or biotinylated

swine anti-rabbit Fab_2 immunoglobulins, respectively [each diluted in PBS containing 10% (vol/vol) normal human serum (PBS-NHS)]. Sections were washed and subsequently incubated for 30 min with HRP-labeled streptavidin-ABC complex, diluted in PBS-NHS, according the manufacturer's instructions. HRP activity was detected by incubating the slides for three minutes with 30 mg of diaminobenzidine (Sigma) in 100 ml of PBS containing 20 μl 30% (vol/vol) H_2O_2. As a control, sections were incubated with an irrelevant mAb of the appropriate subclass.

Double staining. To identify the phenotype of granzyme positive cells a sequential immunohistochemical technique was performed on cryostat sections by combining two indirect techniques using FITC-conjugated and biotinylated mAbs as described by van der Loos et al [27]. Serial (5 μm) cryostat sections were air dried and fixed in acetone for 10 minutes at RT. To block endogenous biotin, present in the renal tissue, slides were incubated with 0.5 mg/ml streptavidin (Zymed, San Fransisco, CA, USA) for 20 minutes, rinsed with PBS and incubated for another 20 minutes with 0.1 mg/ml d-biotin (Sigma). Slides were washed, preincubated with 10% (vol/vol) normal mouse serum for 10 minutes, and then incubated for 60 minutes with FITC-conjugated mAb GrB4 or mAbs for phenotype analysis, combined with biotinylated mAb GrA-8. After a washing step, slides were incubated with 0.3% (vol/vol) H_2O_2, 0.1% (wt/vol) sodium azide in PBS for 20 minutes to block the endogenous peroxidase activity. Then, slides were incubated for 30 minutes with HRP-labeled streptavidin-ABC complex and rabbit anti-FITC immunoglobulins diluted in PBS-NHS. The slides were washed with 50 mM Tris-HCl pH 7.6, 150 mM NaCl, and incubated with AP-conjugated swine anti-rabbit immunoglobulins for 30 minutes. After a final wash step the bound AP activity was detected with naphtol-AS-MX phosphate (Sigma), and Fast Blue BB in 0.2 M Tris-HCl pH 8.5 (20 min, in the dark) yielding a blue color. Endogenous AP activity was blocked by addition of 1 mM levamisole to the reaction medium. HRP activity was detected using H_2O_2 (0.1%) and 3-amino-9-ethylcarbazole (Sigma) yielding a red color. Finally, slides were fixed in 4% (vol/vol) formaldehyde in PBS. As a control, irrelevant biotinylated or FITC labeled mAbs were used or the antibodies were omitted.

Immunohistological evaluation

Every tissue section stained for granzyme A, B was scored independently by two observers who were blinded for the clinical data. The number of positive cells were counted per field in four representative areas by high power field magnification (400×) (0: <5; +: 6–25; ++: 26–50; +++: 51–100 and ++++: >100 positive cells per field).

Results

Granzyme A and B expression in allografts

In Table 1, the data profile of the 10 patients who had an episode of acute cellular rejection is shown. From one patient three biopsies were taken during different rejection episodes. Besides clinical and morphological evidence for an acute cellular rejection, biopsies from two patients (nr. 4 and 7) also showed mild interstitial fibrosis and tubular atrophy as indication of chronic nephropathy. As controls, biopsies or nephrectomy specimens were taken from patients with various renal diseases. Patient 13 had received a renal allograft and suffered from a recurrence of his original kidney disease, focal glomerulosclerosis. His biopsy showed focal glomerulosclerotic changes without any sign of rejection.

Table 2. Number of granzyme A, B or CD3 positive cells per high power field magnification (400×)

Patient	Gran A	Gran B	CD3
1	+++	+++	++++
2	++	++	++++
3	++	+++	++++
4	ND	++	++++
5	++	++	++++
6a	+++	+++	++++
6b	++++	++++	ND
6c	+	+++	++++
7	++	++	+++
8	ND	++	++++
9	0	+	++++
10	++	++	+++
Controls			
11	0	0	0
12	0	0	0
13	+	+	++++
14	0	0	ND
15	0	0	++
16	0	0	++++
17	0	0	++++

The number of positive cells were counted per field in four representative areas (0 < 5; +, 6–25; ++, 26–50; +++, 51–100 and ++++, >100 positive cells per field; ND, not determined).

Serial tissue sections of the biopsies were examined for the presence of granzyme A or granzyme B expressing lymphocytes using monoclonal antibodies reacting with granzyme A or B. The results are summarized in Table 2. In all patients showing signs of allograft rejection, granzyme B positive cells (10 to 200 cells per high power field) were found to infiltrate the renal tissue. Likewise, in nearly all biopsies granzyme A positive cells (25 to 200 cells per high power field) were found, except for patient 9 where no granzyme A positive cells were detected. In general, the number of granzyme A positive cells was comparable with that of granzyme B positive cells. Each of both cell populations showed a similar distribution in the renal tissue. All allografts showed extensive infiltration with CD3 positive lymphocytes (Table 2). No correlation between the severity of histological changes of the allograft and the number of granzyme A, B or CD3 positive cells was found (Table 2). In most cases the granzyme A and B positive cells in the allograft were scattered through the renal tissue although clusters of these cells were also observed in all biopsies. Granzyme positive cells were found in the interstitium, the glomeruli, the tubular walls and in the peritubular capillaries (Fig. 1A). Figure 1B shows a tubule which is infiltrated by a lymphocyte expressing granzyme A. In Figure 1C granzyme B expressing cells are located within the glomerulus. The intensity of the granzyme A and B staining was variable and the granzyme expressing lymphocytes showed a heterogenous morphology. Most granzyme positive lymphocytes exhibited features of blasts but also some small lymphocytes showed cytoplasmic staining for granzymes.

None or very low (patient 13) amounts of granzyme A or B positive cells were found in the renal tissues of the control patients, although in four patients extensive CD3 positive infiltrations were found. Some granzyme A and B positive cells were present in the mononuclear infiltrate found in biopsies from a

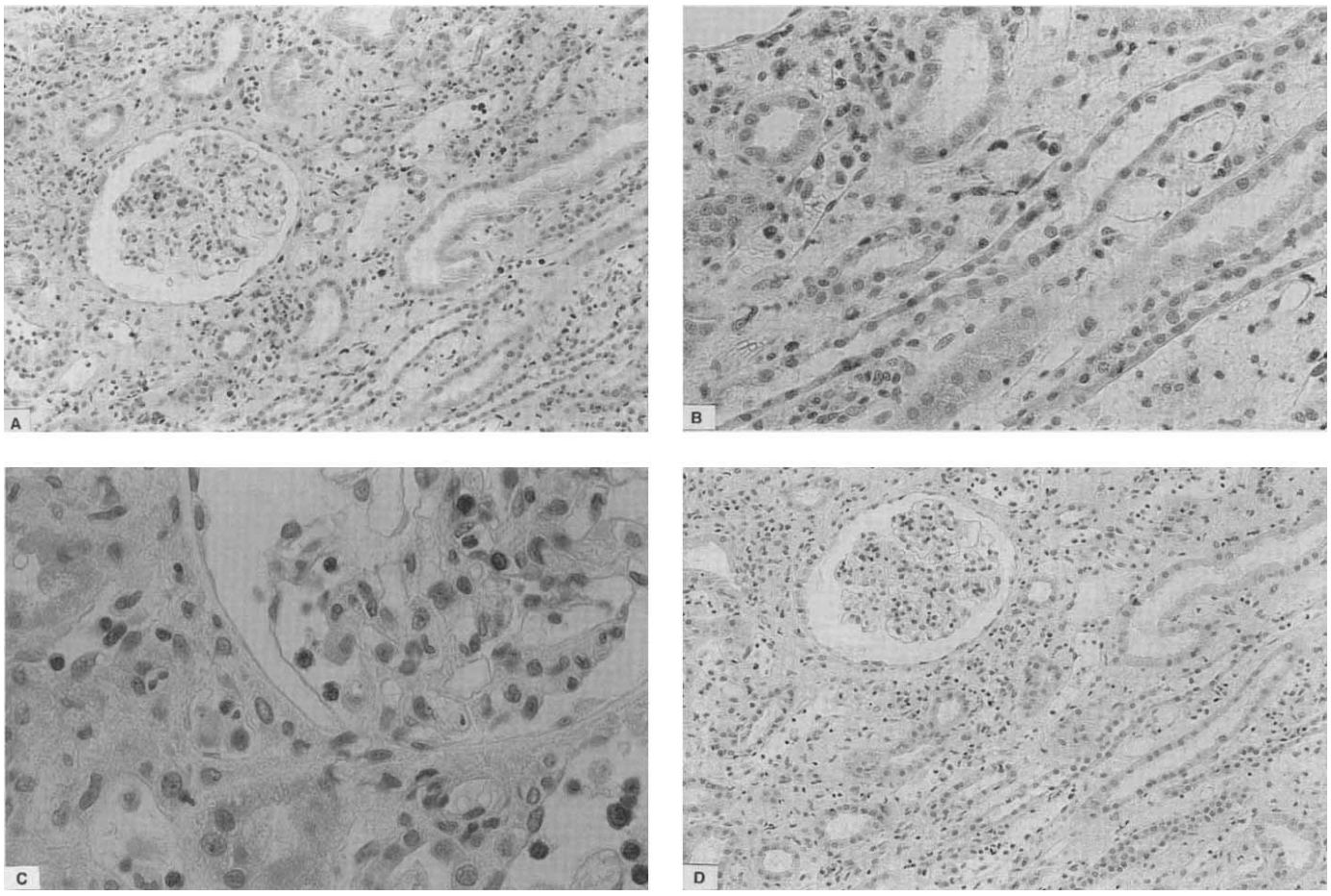

Fig. 1. *Immunoperoxidase staining of granzyme A (***A,** *magnification 175× and* **B,** *magnification 200×) and granzyme B (***C,** *magnification 500×) expressing cells in tissue of a patient during an acute rejection episode.* **D.** A control staining is shown using an irrelevant mAb (magnification 100×).

patient with a renal allograft although no histological evidence was found for rejection. The number of positive cells (<10 per high power field for granzyme A and B) and the amount of granzymes A and B in these cells, as reflected by the staining intensity, in this patient was much lower than the number of granzyme positive cells found in the biopsies showing allograft rejection (with exception of that from patient 9). Also, in this patient no granzyme positive cells were found infiltrating the tubules or glomeruli. One patient with a renal adenocarcinoma (patient 12) showed granzyme A and B expressing cells infiltrating the renal tumor, while no granzyme positive cells were detected in the unaffected renal tissue.

Table 3. Phenotype of granzyme A positive cells in renal allograft biopsies

Patient	CD3	CD4	CD8	CD56
3	<10%	<5%	10%	>85%
4	<15%	<5%	15%	>80%
5	ND	<5%	<5%	>90%
6a	ND	<5%	<5%	>90%
6c	ND	<5%	<5%	ND
8	ND	<5%	<5%	>90%

ND is not determined.

Phenotypic characterization of granzyme positive cells

In six biopsies from five patients a double immunohistochemical technique was used to identify the phenotype of granzyme expressing cells. The results are shown in Table 3. In all cases the majority of granzyme A expressing cells expressed the NK cell marker CD56 on their cell surface. In most patients only a few $CD4^+$ or $CD8^+$ positive cells had detectable granzymes, except for patient numbers 3 and 4, in whom up to 15% of the granzyme A positive cells were $CD8^+$ (Table 3). A subset of NK cells expresses low levels of CD8 on their surface [28]. To exclude that this population accounted for the CD8 positive cells expressing granzymes, we performed double staining with granzyme A and CD3. The equal number of $CD3^+$ and $CD8^+$ granzyme expressing cells in these biopsies indicated that the $CD8^+$ and granzyme positive cells represented activated cytotoxic T lymphocytes. All $CD56^+$ NK cells infiltrating the renal tissue expressed granzyme A. In contrast, most $CD8^+$ cells did not. Figure 2 shows some examples of these studies: granzyme A positive cells double-stained with an anti-CD8 (Fig. 2A) or an anti-CD56 (Fig. 2B) mAb. To determine that granzyme A and B were indeed expressed by the same cells a double staining was also performed using a biotinylated anti-granzyme A and a FITC-labeled anti-granzyme B mAb (Fig. 2C). Most cells positive for granzyme A

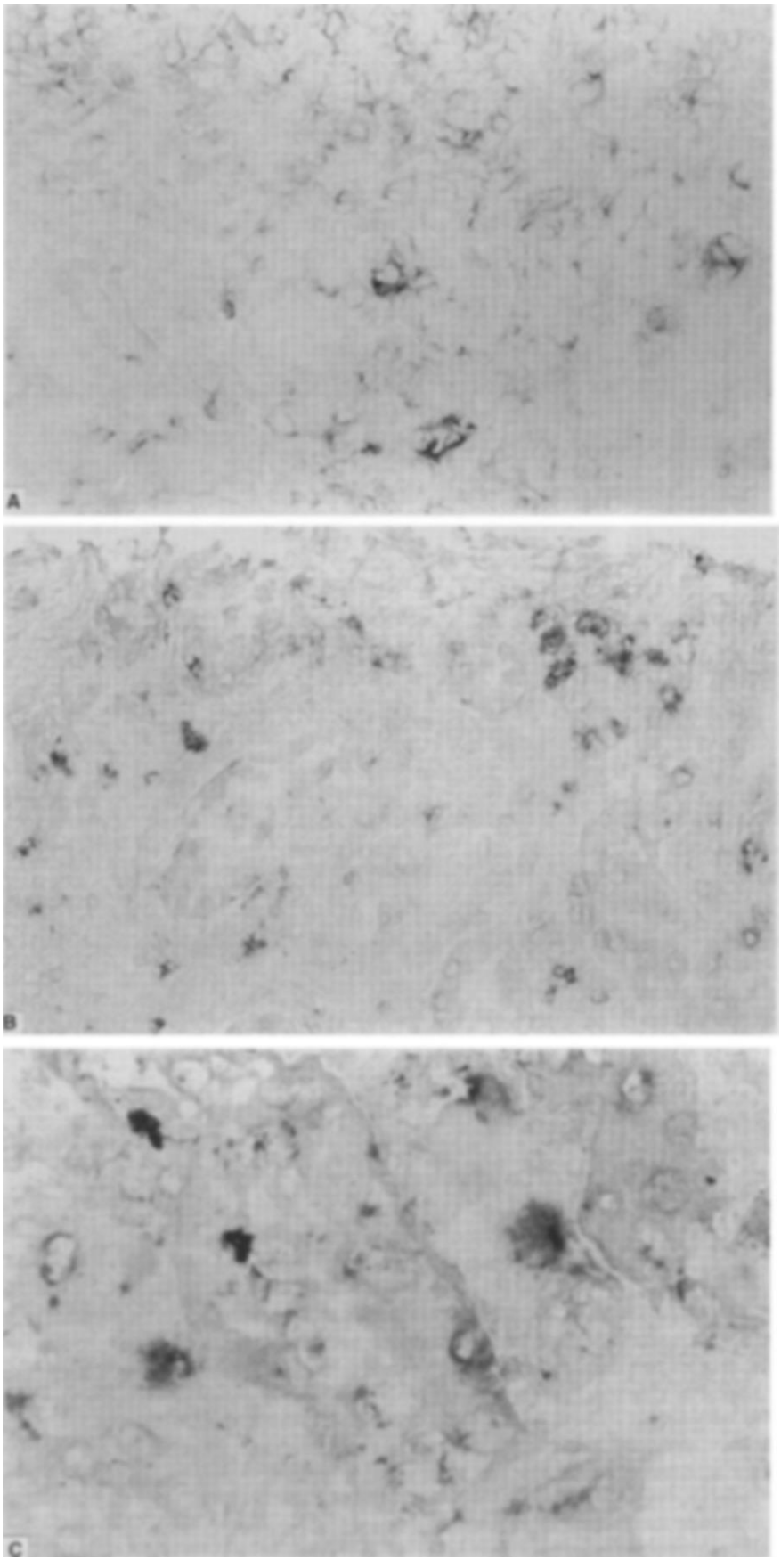

Fig. 2. *Double immunohistochemistry staining of granzyme A expressing cells (peroxidase staining: red) with CD8 (***A,** *magnification 450×), CD56 (***B,** *magnification 350×) or with granzyme B (***C,** *magnification 500×), all detected in blue using alkaline phosphatase staining.*

also stained with the granzyme B mAb, although in some biopsies a small number of cells (<10%) expressed only granzyme B.

The number of granzyme A and B positive cells detected in the TEL fixed, paraffin embedded slides was three to five times higher than that found in the double staining technique on acetone fixed, fresh frozen tissue. This discrepancy probably resulted from a difference in sensitivity between both techniques. Therefore, we also performed a staining with granzyme B and CD3 on thin (2 μm) sequential paraffin embedded tissue sections. From these experiments it was concluded that in the paraffin sections high numbers of granzyme B positive cells were also expressing CD3. Probably the amount of granzyme protein in these cells was too low to be detected in the double staining on fresh frozen tissue. However, in this way granzyme positive cells were also detected which were CD3-negative. This latter is illustrated in Figure 1C, where a glomerulus is infiltrated by numerous granzyme B positive cells while the same area contains hardly any $CD3^+$ positive cells (Fig. 1D).

Discussion

Although the role of granzymes in the cytotoxic process is still not elucidated, expression of these proteins by NK cells and cytotoxic T lymphocytes correlates with the cytotoxic potency of these cells *in vitro* [15–17]. The availability of specific mAbs reacting with granzymes facilitates the evaluation of the role of these cells *in vivo*. In this study we used these mAbs to study granzyme expression by cells in renal allografts from patients suffering from an acute cellular rejection. In only one patient with histological signs of rejection a relatively low number of the infiltrating cells expressed granzyme B, whereas no granzyme A positive cells were found. The other patients with allograft rejection, were all characterized by having high numbers of granzyme A as well as granzyme B protein expressing lymphocytes infiltrating the renal allograft tissue. In spite of the presence of a marked inflammatory infiltrate, no granzyme positive cells were found in several inflammatory, not transplant-related, renal diseases. Only in one patient, with a renal adenocarcinoma, granzyme positive cells were found in the tumor, which is consistent with a role of cytotoxic lymphocytes in the immunologic response to tumor cells. Therefore, granzyme expression by lymphocytes infiltrating the kidneys seems to be rather specific for allograft transplant rejection. Whether this expression is specifically related to an rejection episode is more difficult to establish since no tissue specimens, except for one, were available from patients with a renal transplant without a rejection episode. Such specimens are, for ethical reasons, hard to obtain. In one specimen from a patient with a renal transplant without rejection, we found some granzyme A and B positive cells in the absence of histological signs of rejection. However, the number and intensity of granzyme positive cells was much lower compared to that of the patient group. This may be a reflection of the existence of a 'low grade' of immune activity in the allograft, independent on the presence of rejection episodes. Although a good correlation existed in the patient group studied between a clinical score for acute rejection and either the BANFF classification or semiquantitative amount of granzyme A and B expression, we think that study of a much larger group of patients is required to establish such a possible relationship with certainty.

Our results extend a recent study in which mRNA of granzyme B gene was determined in patients with renal allograft rejection using the PCR technique. Granzyme B gene expression was almost exclusively confined to biopsies from patients with acute cellular rejection compared to non-rejected allografts, whereas no significant differences were found between rejection and non-rejection samples regarding the gene transcripts of IL-1β, IL-2, IL-6, TNF-α and IL-2 receptor beta [24]. Similarly, our results indicate that expression of granzyme B proteins, and in addition also that of granzyme A, is associated with cellular allograft rejection. Moreover, perforin expressing lymphocytes could be detected by immunohistochemical methods in a small group of renal allograft patients showing acute allograft rejection [29, 30], although others have shown that intragraft perforin gene expression was found to be a less sensitive marker compared to that of granzyme B gene expression [31].

Phenotypic analysis of cells infiltrating allograft tissues (for example, the CD4:CD8 ratio) does not always provide reliable information on the functional status of the graft itself [32]. Although florid rejection is readily identified by histological parameters, virtually every allograft is infiltrated to some degree by host lymphocytes. Furthermore, the cytotoxic cells present in allograft tissues do not always represent functional killers. Expression of cytolytic mediators such as granzyme A and perforin is suppressed by corticosteroids and cyclosporin A [33, 34]. Heart allografts showed minimal granzyme A positive cells after cyclosporin A treatment, whether cellular infiltrates developed or not [34]. All patients in our study received immunosuppressive drugs consisting of corticosteroids as well as cyclosporin A. In spite of this medication granzyme positive cells were found in all patients, indicating that the immunosuppressive medication in these patients could not prevent the transcription and translation of granzymes, apparently resulting in functional active cytotoxic cells. At present we are investigating whether successful immunosuppressive treatment of renal allograft rejection is followed by decreased expression of granzymes.

It is generally believed that rejection episodes of renal allotransplants are mediated by the cytotoxic attack of host lymphocytes via T cell receptor recognition of donor alloantigens, that is, MHC class I and II. However, several cell adhesion molecules (ICAM-1, VCAM-1) are increased on the vascular endothelium and on the tubular system during allograft rejection [35, 36]. The increase of these molecules enhances the susceptibility to T cell and NK cell mediated lysis [1, 37, 38]. In agreement herewith, most biopsies contained $CD3^+$ and $CD3^-$ granzyme positive cytotoxic cells infiltrating the tubular walls, the peritubular capillaries and the glomeruli. Previously this was also observed for perforin positive cells in renal allograft rejection [30].

The granzyme positive infiltrating cytotoxic cell population consisted predominantly of $CD3^-CD56^+$ NK cells and also in some patients $CD3^+$ $CD8^+$ CTL, as determined by double immunostaining techniques on fresh frozen tissue. This would suggest that NK cells are the predominant cells expressing granzymes in renal allograft rejection. However, the granzyme staining on the paraffin embedded tissue slides appeared to be more sensitive, and we estimated that the phenotype of about only 20% of the cells positive for granzymes could be determined by double staining techniques. To establish the phenotype of the other granzyme-positive cells we performed CD3 and granzyme B staining of serial paraffin-embedded sections. The results showed that these granzyme positive cells were mainly CD3 positive and probably represented $CD3^+$ $CD8^+$ CTL. These results indicate

that during rejection of renal allotransplants the cells producing the highest level of granzyme proteins are activated NK cells, and that CTL express lower amount of these mediators.

These observations suggest a strong local production of IL-2 leading to the activation of CTL and NK cells into activated killer cells (LAK), which may mediate non-MHC restricted cellular cytotoxicity [37, 39]. LAK cells have been isolated from renal allograft tissue from patients with acute cellular rejection and are able to kill both glomerular and tubular cells grown from the same nephrectomy specimen without showing MHC antigen restriction [39]. Interestingly, these LAK cells showed a similar lytic potential for renal cells obtained from third party renal tissue [39]. Even LAK cells obtained by stimulating peripheral blood mononuclear cells with high dose of IL-2 were able to lyse normal kidney cells [39–41]. *In vitro*, LAK cells not only mediate lysis of tubular cells, but can also impair the function of kidney cell monolayers by destroying the transepithelium resistance in the abscence of cell lysis [40]. These *in vitro* phenomena may occur *in vivo*, as we observed activated cytotoxic, that is, granzyme positive, cells infiltrating the renal epithelial cells of the tubular wall. Although the role of the different granzymes in the cytotoxic process is still not elucidated [42–44], granzymes can mediate inflammatory functions such as the breakdown of extracellular matrix proteins and vascular basement membranes [45, 46], or activation of other proteinases such as pro-urokinase [47]. The release of these proteases during the rejection episode could result in the destruction of the tight junctions between the tubular cells or in the breakdown of other renal tissue components.

In conclusion, granzyme proteins are expressed by infiltrating NK cells and CTLs in kidney allografts during acute cellular rejection episode as detected by immunohistochemistry. Their role in the pathogenesis of graft injury and their relevance in predicting or monitoring graft rejection remains to be established.

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Reprint requests to Prof. dr. C.E. Hack, c/o Publication Secretariat, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, 1066 CX, Amsterdam, The Netherlands.

Appendix Abbreviations

Abbreviations are: AP, alkaline phosphatase; CTL, cytotoxic T lymphocyte; FITC, fluorescein isothyocyanate; HRP, horseradish peroxidase; mAb, monoclonal antibody; MHC, major histocompatibility complex; NK, natural killer; PBS, phosphate-buffered saline, pH 7.4; PBS-B, PBS-1% (wt/vol) bovine serum albumin; PBS-NHS, PBS-10% (vol/vol) normal human serum; PCR, polymerase chain reaction; RT, room temperature; TEL, Tellyesniczky.

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